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Natural fumigation as a mechanism for volatile transport between flower organs

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Plants synthesize volatile organic compounds (VOCs) to attract pollinators and beneficial microorganisms, to defend themselves against herbivores and pathogens, and for plant-plant communication. In general, VOCs accumulate in and are emitted from the tissue of their biosynthesis. However, using biochemical and reverse genetic approaches, we demonstrate a new physiological phenomenon: inter-organ aerial transport of VOCs via natural fumigation. Before petunia flowers open, a tube-specific terpene synthase produces sesquiterpenes, which are released inside the buds and then accumulate in the stigma, potentially defending the developing stigma from pathogens. These VOCs also affect reproductive organ development and seed yield, which are previously unknown functions of terpenoid compounds.

Decades of research have uncovered essential roles of volatile organic compounds (VOCs) in plant fitness. VOCs are lipophilic, low molecular weight compounds with high vapor pressure at ambient temperatures, which have been shown to be instrumental in plant–plant, plant–animal, and plant–microbe interactions[1,2]. VOCs are chemically diverse but are mainly classified as terpenoids, phenylpropanoids and benzenoids, and fatty acid and amino acid derivatives. They are typically released from the tissue(s) of origin directly into the surrounding environment, usually with low accumulation within the synthesizing tissues. Larger pools of VOCs may also be sequestered in the tissue of biosynthesis, either physically in specialized structures like trichomes[3], or chemically via VOC modifications that prevent their volatilization[4,5]. VOCs have been shown to be an essential part of the plant defense response, including plant priming, in which attacked plants emit compounds that are perceived by leaves of neighboring plants without detectable accumulation within the receptive tissues[6]. Although volatile signaling between leaves of the same or nearby plants has been reported, the involvement of VOCs in inter-organ communication remains largely unexplored.

Of all plant organs, flowers emit the highest levels of VOCs. Many of these VOCs are used either to attract specific pollinators or repel antagonist insects[7–9], thus ensuring plant reproductive and evolutionary success. Flowers are also an ideal habitat for and highly susceptible to pathogens and florivores owing to their high nutrient content and low lignification of cell walls that serve as a physical barrier against penetration. Within the flower, stigmas are especially susceptible to pathogens. Their moist and nutritive environment, which promotes germination and growth of pollen grains, can also facilitate growth of microorganisms[10]. However, the molecular mechanisms responsible for protection of reproductive organs, and subsequent overall plant survival in natural ecosystems, still remain to be determined.

Petunia hybrida flowers are an excellent model system for investigating the biosynthesis, regulation, and emission of plant VOCs. Although petunia flowers produce predominantly phenylpropanoid and benzenoid compounds, low levels of two sesquiterpene compounds, germacrene D (**1**) and β-cadinene (**2**) (cadina-3,9-diene), have also been detected in in vitro headspace of $CaCl_2$ extracts[11]. In an attempt to understand the biosynthesis of these terpenoid volatiles and their role(s) in petunia flowers, we identified and characterized terpene synthases (TPSs) responsible for terpenoid production. Moreover, we discovered natural fumigation (that is, gas treatment of an enclosed space) as a mechanism for directional inter-organ terpenoid transport, and a previously unidentified function for volatile terpenoids in the development of reproductive organs.

Results

Petunia TPSs and the fate of their products. We used targeted metabolite profiling of volatiles present in petunia floral organs and detected two additional sesquiterpenes, bicyclogermacrene (**3**) and germacrene D-4-ol (**4**), and the monoterpene geraniol (**5**) along with previously detected terpenes, germacrene D and β-cadinene (Supplementary Fig. 1). The highest levels of sesquiterpenes were found in pistils, with significantly lower levels in other floral organs, including stamens, tubes, and corollas (Supplementary Fig. 1). No sesquiterpene glucosides were detected in any floral tissue. By contrast, geraniol and its glucoside were found only in pistils and were absent in other analyzed tissues (Supplementary Figs. 1,2a). In addition, terpene levels did not display any time-dependent changes throughout the day (Supplementary Fig. 2b).

To identify genes that encode enzymes that are responsible for terpene production, we searched petunia flower RNA-seq data sets[12] for TPS genes. This search resulted in the identification of four putative TPS genes, designated *PhTPS1* to *PhTPS4*, numbered according to their expression levels (Supplementary Fig. 3). Phylogenetic analysis revealed that PhTPS1, PhTPS3, and PhTPS4 all belong to the TPS-a clade, which contains most of the characterized sesquiterpene synthases[13], whereas PhTPS2 belongs to the TPS-b clade, which includes monoterpene synthases (Supplementary Fig. 4). Indeed, expression of *PhTPS2* in *Saccharomyces cerevisiae* resulted in geraniol production (Supplementary Fig. 5a). Furthermore,

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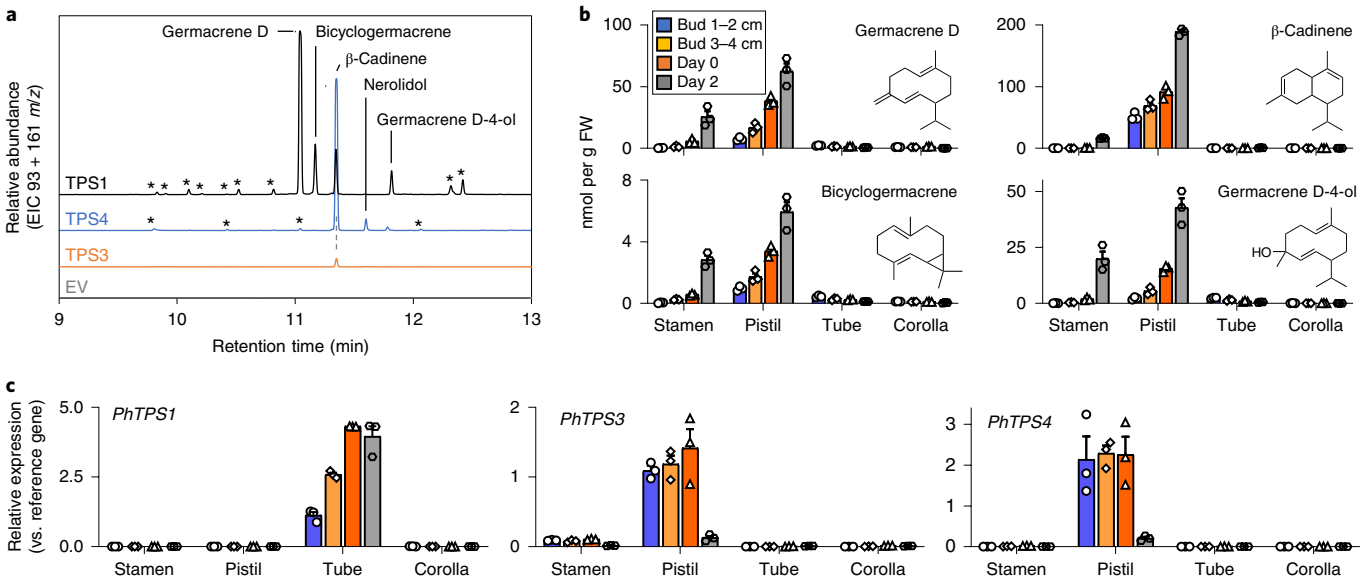

Fig. 1 | Characterization of sesquiterpene synthases expressed in petunia flowers. a), Products of PhTPS1, PhTPS3, and PhTPS4 or empty vector (EV) expressed in yeast strain WAT11. GC-MS chromatograms of volatiles emitted from yeast cultures are presented as total extracted ion current (EIC, *m/z* 93 + 161). Sesquiterpenes were identified by comparison of their mass spectra to the NIST library. Germacrene D was confirmed by comparison with an authentic standard. Asterisks represent unidentified putative sesquiterpenes. GC-MS chromatograms are representative of three independent experiments. **b**,**c**, Internal pools of sesquiterpenes (**b**) and relative transcript levels of *PhTPS1*, *PhTPS3*, and *PhTPS4* (**c**) in petunia stamen, pistil, tube, and corolla over flower development (1–2 cm buds, 3–4 cm buds, flower day 0 before anthesis, and flower day 2 after anthesis) determined by GC-MS (**b**) and by qRT-PCR (**c**), respectively. Internal pools were quantified based on the ratio of the integrated peak area of terpenoids relative to the IS peak area and normalized to the weight of the tissue. *PhPP2A* was used as a reference gene in qRT-PCR. Data are means ± s.e.m. ($n = 3$ biological replicates).

PhTPS2 transcript levels measured by quantitative reverse transcription PCR (qRT-PCR) were found predominantly in petunia pistils and correlated positively with geraniol accumulation over the course of flower development (Supplementary Fig. 5b,c).

To test whether the petunia sesquiterpene synthase candidates are responsible for biosynthesis of sesquiterpenes detected in flower organs, product specificities of PhTPS1, PhTPS3, and PhTPS4 were determined by analyzing the terpenoid profiles in yeast expressing each of the enzymes (Fig. 1a). PhTPS1 produced a dozen sesquiterpenes, of which the major products were germacrene D, bicyclogermacrene, β-cadinene and germacrene D-4-ol. PhTPS4 generated mainly β-cadinene with a small amount of nerolidol, and PhTPS3 produced only a small amount of β-cadinene (Fig. 1a).

Of sesquiterpenes detected in pistils, β-cadinene was the most abundant compound. Its accumulation, as well as that of other sesquiterpenes, was developmentally regulated with the highest levels on day 2 after floral opening (post anthesis) (Fig. 1b). Despite the fact that all three petunia sesquiterpene synthases produce β-cadinene (Fig. 1a), its accumulation in pistils correlated spatially only with *PhTPS3* and *PhTPS4* expression (Fig. 1b,c). Unexpectedly, germacrene D, bicyclogermacrene and germacrene D-4-ol, the sesquiterpenes produced only by PhTPS1, accumulated mainly in the pistil (Fig. 1b) at ratios similar to their production by recombinant protein and their occurrence in tubes (Supplementary Fig. 6), even though *PhTPS1* expression occurred almost exclusively in the tube (Fig. 1c). Interestingly, *PhTPS1* mRNA was not evenly distributed across the tube. The maximum expression occurred mainly at the top of the tube just below the unexpanded corolla (Supplementary Fig. 7a), the part of the tube in closest proximity to the developing stigma (Supplementary Fig. 7b) where PhTPS1 products accumulate the most (Supplementary Fig. 7c). Moreover, *PhTPS1* expression was developmentally regulated, peaking 1 d ahead of sesquiterpene levels in the pistil (Fig. 1b and Supplementary Fig. 7d), as has been observed for other VOC biosynthetic genes[14].

Consistent with expression data, crude protein extracts prepared from pistils produced only β-cadinene (and not the other sesquiterpenes) when incubated with *e,e*-farnesyl diphosphate (FPP), the sesquiterpene precursor (Supplementary Fig. 8a,b). Similarly, activity for germacrene, bicyclogermacrene, and germacrene D-4-ol formation was detected only in the crude protein extracts prepared from the top of the tube (Supplementary Fig. 8c), where expression of *PhTPS1* was the highest (Fig. 1c and Supplementary Fig. 7a).

Inter-organ transport of terpenes via bud headspace. The absence of *PhTPS1* expression and TPS activity that is capable of producing germacrene D, bicyclogermacrene, and germacrene D-4-ol in pistils along with simultaneous accumulation of these terpenoids in the stigma suggests that PhTPS1 products detected in the stigma originate in the tube. Thus, we hypothesized that these sesquiterpenes are emitted from the tube into the bud's headspace and accumulate in reproductive organs before flower opening, probably protecting the nutrient-rich pistil from attacking pathogens and florivores via natural fumigation.

To test this aerial transport hypothesis, we first compared the emission rates of PhTPS1 products from the inner (adaxial) and outer (abaxial) surfaces of the petunia tube by using direct-contact sorptive extraction (DCSE) with polydimethylsiloxane coated stir bars (Twisters)[15]. We found that approximately 65 to 75% of each compound was emitted from the inner side of the tube (Supplementary Fig. 9), suggesting a directional release of terpenoids into the headspace of closed buds. Second, tubes were removed from flower buds 4 d before anthesis, and accumulation of PhTPS1 products was analyzed in pistils at day 1 post anthesis (Supplementary Fig. 10a). In contrast to control flowers, germacrene D, bicyclogermacrene, and germacrene D-4-ol were almost completely absent in pistils from flowers grown without tubes (Fig. 2a). However, these pistils still accumulated geraniol and β-cadinene, most probably because of the

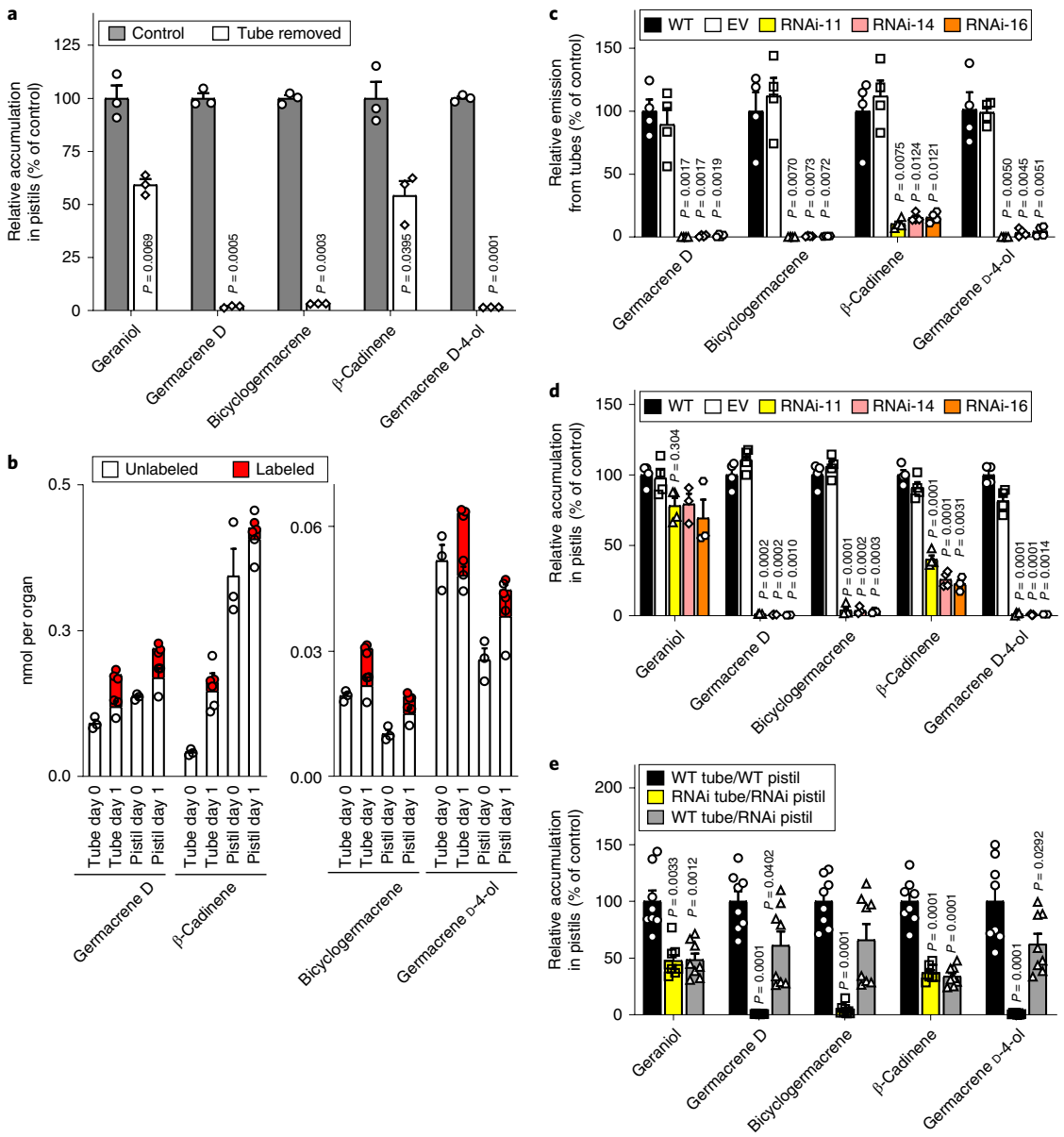

Fig. 2 | Analysis of inter-organ transport of PhTPS1 products in petunia buds. a, Effect of flower tube on accumulation of terpenoids in pistils. Terpenoids were analyzed in pistils on day 1 post anthesis from intact flowers (control) and from flowers from which tubes were removed 4 d before anthesis. Data are means ± s.e.m. ($n = 3$ biological replicates). **b**, GC-MS analysis of sesquiterpenes produced by petunia tubes fed with [2-^{13}C]-mevalonolactone for 24 h. Amounts of unlabeled and labelled sesquiterpenes were quantified with the specific ions 161 m/z and 162 + 163 m/z (M + 1 and M + 2), respectively. Data are means ± s.e.m. ($n = 3$ biological replicates). **c**,**d**, Effect of *PhTPS1* RNAi downregulation on terpenoid emission from tubes over 24 h beginning on day 0 before anthesis (**c**) and their accumulation in pistils on day 1 post anthesis (**d**). WT, wild-type control; EV, empty-vector control; and *PhTPS1* RNAi lines. Data are means ± s.e.m. ($n = 4$ biological replicates). **e**, Effect of gas phase complementation by wild-type tubes on internal pools of mono- and sesquiterpenes in *PhTPS1* RNAi-11 pistils. Terpenoids were extracted from pistils after 24 h of complementation and analyzed by GC-MS. Data are means ± s.e.m. ($n = 8$ biological replicates). Experimental setups for **a**, **b** and **e** are illustrated in Supplementary Fig. 10. Significant P values ($P < 0.05$), as determined by two-tailed paired t-test relative to control, are shown.

respective activities of PhTPS2 and PhTPS3 and/or PhTPS4 present in this organ (Fig. 2a).

Third, we assessed the movement of PhTPS1 products from the tube to the pistil by feeding only the tubes with a stable isotope-labeled precursor of sesquiterpenes and tracking the fate of labeled products in detached pistils (Supplementary Fig. 10b). Feeding of petunia tubes from day 0 flowers with [2-^{13}C]-mevalonolactone, a precursor of FPP, led to accumulation of labeled sesquiterpenes not only in tubes but also in pistils (Fig. 2b), despite the fact that pistils were detached and protected from contact with [2-^{13}C]-mevalonolactone by microvials (Supplementary Fig. 10b). These results provide direct evidence for gas phase transmission of stable isotope labelled terpenoids between these flower organs (Fig. 2b).

Lastly, we generated transgenic petunia plants with constitutive RNA interference (RNAi) downregulation of *PhTPS1* expression (Supplementary Fig. 11). The three independent lines with *PhTPS1* transcript levels reduced by 92 to 94% barely emitted PhTPS1 products from tubes (Fig. 2c) and had drastically reduced accumulation of these sesquiterpenes in pistils (Fig. 2d). To test directly for gas

phase transport, we performed a complementation experiment in which *PhTPS1* RNAi pistils were placed within wild-type tubes for 24 h (Supplementary Fig. 10c). Volatiles released from the wild-type tubes were indeed sufficient to restore the accumulation of missing sesquiterpenes in transgenic pistils (Fig. 2e). Analysis of expression of other *PhTPSs* in pistils of transgenic plants revealed an unexpected decrease in *PhTPS4* expression, whereas *PhTPS2* and *PhTPS3* were unaffected (Supplementary Fig. 12). Although the reasons for this reduction are unknown, it correlated with the observed decrease in β-cadinene levels in transgenic pistils (Fig. 2d), an effect that persisted even upon complementation with wild-type tubes (Fig. 2e).

Sesquiterpene fumigation alters pistil microbiome. Flower terpenoids are known to affect the growth of bacterial communities and to protect reproductive organs from microbial pathogens[16], thus we hypothesized that terpene fumigation might affect the bacterial and fungal communities present on the pistil surface. Therefore, we characterized microbiome of wild-type and transgenic pistils. Fungal operational taxonomic units (OTUs) were not detected in pistils of either wild-type or *PhTPS1* RNAi flowers. Bacterial diversity and number of reads were found to be very low (only 7 OTUs) on the surface of pre-anthesis stigma and even lower on day 2 post anthesis (Supplementary Fig. 13a and Supplementary Dataset 1), demonstrating a low bacterial density on petunia stigmas in general. However, lack of sesquiterpene fumigation on pistils of *PhTPS1* RNAi flowers resulted in a statistically significant increase in the most abundant bacterial OTU in our samples, belonging to the family Pseudomonas, relative to their level in wild-type pistils (Supplementary Fig. 13b). Furthermore, bacterial community composition responded to time after anthesis and plant genotype (wild type or transgenic, Supplementary Fig. 13c). These results suggest that fumigation affects bacterial growth on stigmas, and support the role of terpenoids in shaping bacterial communities of petunia reproductive organs[17].

Floral fumigation affects pistil growth and seed yield. Although there is no detectable *PhTPS1* expression in developing pistils, downregulation of *PhTPS1* transcript levels and the corresponding loss of sesquiterpene fumigation had a striking effect on pistil development. *PhTPS1* RNAi pistils had significantly lower weights than wild-type pistils (~78 to 86% of wild-type flowers) (Fig. 3a and Supplementary Fig. 14a,b) and smaller stigmas (Supplementary Fig. 14d–f). Transgenic flowers also had slightly decreased style length and reduced diameter (Supplementary Fig. 14c,e,f). Further experiments showed that pistil size phenotype is independent of pistil genotype and instead depends on tube genotype. In vitro growth of *PhTPS1* RNAi pistils within wild-type tubes (Supplementary Fig. 10d) resulted in recovery of the pistil size phenotypes (Fig. 3b and Supplementary Fig. 14g). This result directly confirmed that volatiles that are produced by PhTPS1 and fumigated from the tube regulate pistil growth. Moreover, wild-type pistils grown within *PhTPS1* RNAi tubes exhibited a similar reduced pistil size phenotype to transgenic pistils (Fig. 3b and Supplementary Fig. 14g), which further supports the conclusion that terpenoids that are produced by PhTPS1 in tubes are required for normal pistil development.

Disruption of *PhTPS1* expression also affected the seed yield but not the seed weight (Fig. 3c). *PhTPS1* RNAi flowers produced up to 33% fewer seeds than wild-type flowers (Fig. 3c) without any effect on pollen tube growth through the pistils after pollination (Supplementary Fig. 15). These data reveal a role of aerial transport of sesquiterpenes in pistil development and seed yield, and thus successful reproduction (or fitness).

Pistils contain more cuticular waxes than tubes. In general, VOCs (including sesquiterpenes) are lipophilic and can readily partition from air spaces into the epicuticles of plants[18]. Therefore, the directional transport of the fumigated sesquiterpenes from the tube and their accumulation in the pistil, rather than other tissues of the bud, may be the result of stigma surface physicochemical properties. Indeed, comparative analysis of waxes revealed that pistils contain a significantly higher level of waxes than tubes (Supplementary Fig. 16a), which could cause the preferential adsorption of sesquiterpenes to the pistil surface. Pistils that were exposed to artificial fumigation with β-caryophyllene showed time-dependent accumulation (Supplementary Fig. 16b) and accumulated higher levels of exogenous sesquiterpene than the stamen and tube, further demonstrating the pistil's ability to preferentially trap volatiles (Supplementary Fig. 16c).

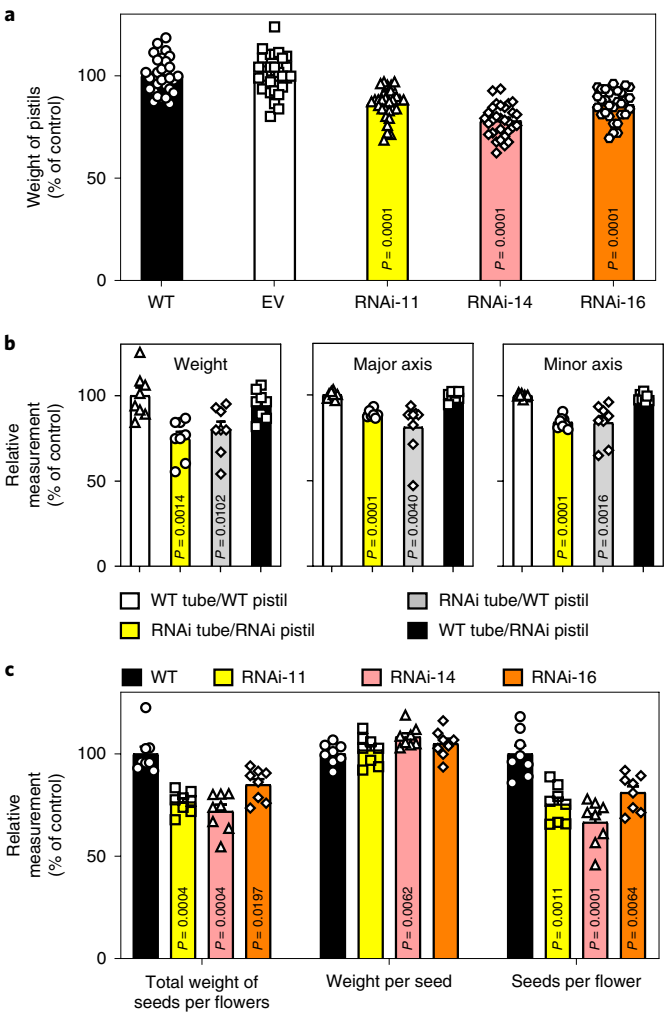

Fig. 3 | Effect of sesquiterpene fumigation on pistil development and seed yield. a, Weight of pistils from WT, EV control and *PhTPS1* RNAi lines on day 1 post anthesis. Data are means ± s.e.m. ($n = 30$ biological replicates). **b**, Complementation via gas phase of in vitro growth of *PhTPS1* RNAi-11 pistils with WT tubes. Experimental setup is illustrated in Supplementary Fig. 10d and described in the Methods. After 4 d of complementation, weight, and major and minor axes of stigma were measured. Data are means ± s.e.m. ($n = 8$ biological replicates). **c**, Effect of *PhTPS1* RNAi downregulation on seed production. Total weight of seeds per flower, weight per seed and number of seeds per flower were measured in WT and *PhTPS1* RNAi transgenic lines. Data are means ± s.e.m. ($n = 8$ biological replicates). Significant *P* values ($P < 0.05$), as determined by two-tailed paired *t*-test relative to control, are shown.

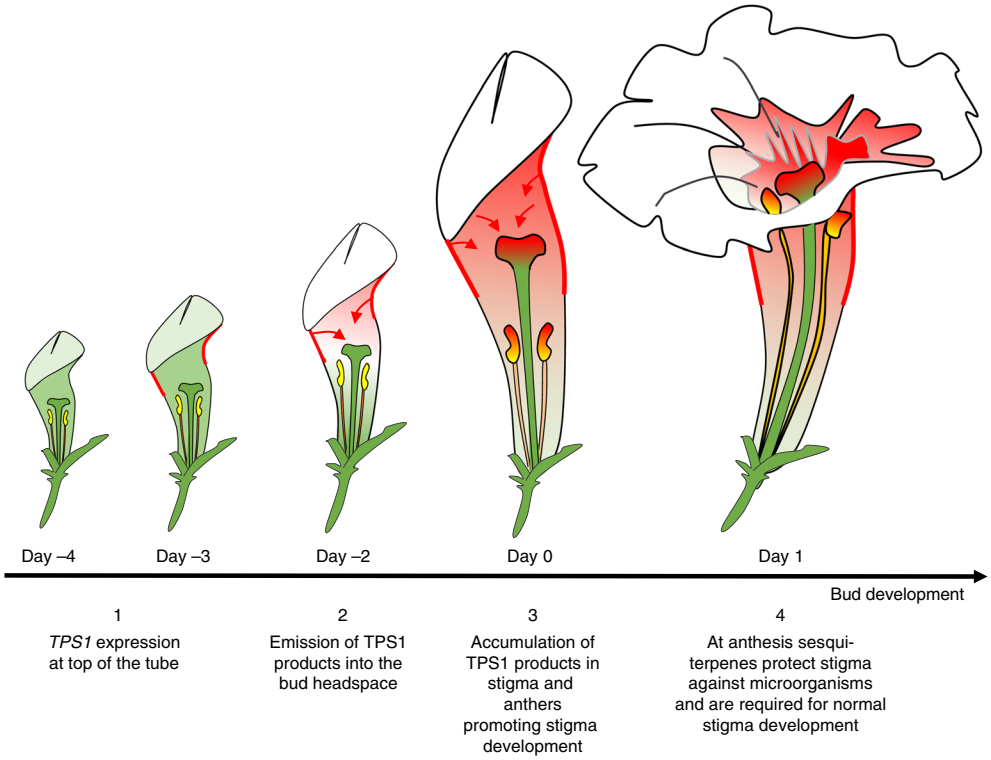

Fig. 4 | Proposed fumigation model. Scheme showing the sesquiterpene fumigation process during petunia bud development. At young bud stage, *PhTPS1* is expressed (shown in red) at the top of the tube below the corolla (step 1). PhTPS1 sesquiterpene products are emitted in the bud headspace (red area and arrows, step 2) and are then absorbed and accumulated by the stigma and anthers (step 3). This process ensures normal pistil development and protects reproductive organs against microorganisms when the flower opens (step 4).

Discussion

Volatile terpenoids constitute the majority of plant VOCs and are dominated by mono- and sesquiterpenes. As a part of plant defense, they not only repel and intoxicate attacking herbivores above and below ground, and mediate plant–insect and plant–microbial interactions, but are also instrumental in plant–plant communication, including priming[1,19,20]. Plant–plant communication via VOCs includes intra- and inter-specific signaling as well as within-plant self-signaling between different branches or adjacent leaves. In all of these cases, plant VOCs are first released into the atmosphere to create a diffuse signal that is then perceived by leaves of the same or neighboring plants. Although it is clear that receiving plants perceive and react to VOC signal(s), no accumulation of free VOCs has been detected inside recipient tissues. In contrast, in petunia pistils we found high accumulation of sesquiterpenes, which were produced and released by surrounding tubes. This occurs via natural fumigation in the enclosed space of floral buds before flower opening (Fig. 4).

The fumigation of pistils with sesquiterpenes may allow plants to protect their reproductive organs against microorganisms. For example, *Arabidopsis thaliana* stigmas produce a sesquiterpene, (*E*)-β-caryophyllene, to inhibit the growth of a *Pseudomonas syringae*, a pathogen that causes seed defects[17]. In general, VOCs have been shown to affect the growth of microorganisms associated with plant tissues[16,21]. Our data suggest that the fumigation of the pistils reduces the density of bacteria colonizing petunia pistils (Supplementary Fig. 13), thereby potentially protecting this tissue against pathogens. Moreover, recent studies have demonstrated that floral microbes can affect pollinator behavior by altering the floral scent emission[22,23]. Therefore, mechanisms that prevent the colonization of important reproductive tissues by bacteria may be beneficial for plant reproduction even beyond their function in protection against pathogens.

As the sesquiterpenes produced by TPS1 are beneficial to the development of the stigma, it is not clear why pistils have to be fumigated by tubes rather than synthesizing these compounds de novo by themselves, as occurs for β-cadinene (Figs. 1a,b,2a). However, as the absence of tube-produced sesquiterpenes has detrimental effects on pistil development and seed yield (Fig. 3 and Supplementary Fig. 14), it is likely that aerial transmission of volatiles from tubes might serve as a signal, allowing coordination of growth of different floral organs. In outcrossing species such as *Petunia hybrida*, regulation of pistil development by volatile terpenoids from surrounding tube could serve as a mechanism to coordinate the timing of pistil maturation with petal development to ensure that the stigma is receptive when the flowers are most likely to attract pollinators. Such 'hormone-like' action would be analogous to the well-established ethylene signaling that coordinates senescence of the petal tissue with successful pollination[24,25].

There is a plethora of examples in which volatile signals serve as developmental cues. Ethylene, the function of which is not limited to flowers, serves as a key growth regulator that impacts leaf, root, shoot, and fruit development[26]. Many of these ethylene roles overlap with those of another volatile hormone, methyl jasmonate. Methyl jasmonate, although generally considered a defense compound owing to its involvement in responses to biotic and abiotic stresses, has a wide-ranging influence on a variety of developmental processes, including seed germination, root growth, fruit ripening, and senescence[27,28]. Another example of mobile signal is methyl salicylate, which has an important role in plant defense[19,29].

Our results show that volatile sesquiterpenes are necessary for optimal pistil growth (Fig. 3a,b and Supplementary Fig. 14) and represent the first example of plants using their own sesquiterpenes to regulate flower development. This is consistent with previous results showing that terpenoid compounds can potentially influence

plant growth. However, in these cases terpenoids were produced by micorrhizal fungi[30]. β-Caryophyllene produced by *Fusarium oxysporum* was shown to increase root and shoot length, as well as the fresh weight of lettuce (*Lactuca sativa*) seedlings[31]. Lateral root growth-promoting activity was also reported for another sesquiterpene, (-)-thujopsene, released by *Laccaria bicolor*, in grey poplars and *Arabidopsis*[32].

Flower development has been studied extensively from a molecular genetic perspective[33,34]. However, the function of terpenoids in this process has been overlooked, probably owing to their low levels before flower opening. It is possible that the directional inter-organ transport of volatile terpenoids and subsequent hormone-like effects on pistil growth and seed yield observed in this study are linked to floral morphology. Further studies are required to assess whether natural fumigation is conserved in flowering plants, to uncover the mechanisms involved, and to determine its evolutionary advantage in plant reproduction.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41589-019-0287-5.

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Author contributions

B.B. and N.D. conceived the study. B.B., S.R., J.Y., J.H.L., R.R.J., S.A.K., J.A.M., and N.D. planned experiments. B.B. performed metabolic profiling, identification, and characterization of TPSs, analysis of TPS activities in plants, stable isotope labeling, expression analysis, generation of transgenic plants and their analysis, and complementation experiments. S.R. performed wax analysis. J.Y. performed microscopy analysis. K.M.P.C. analyzed seed yield. J.H.L. performed complementation experiments, expression analysis of TPSs in transgenic plants, and metabolic profiling. R.R.J. performed microbiome analysis. B.B., S.R., J.Y., K.M.P.C., J.H.L., R.R.J., S.A.K., J.A.M., and N.D. analyzed and interpreted data. S.A.K., J.A.M., and N.D. supervised the study. B.B., J.H.L., and N.D. wrote the paper. All authors read and edited the manuscript.

Competing interests

The authors declare no competing interests.

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Methods

Plant materials and growth conditions. *Petunia hybrida* cv. Mitchell diploid (W115; Ball Seed Company) wild-type and *PhTPS1* RNAi transgenic plants were grown under standard greenhouse conditions as described previously[35]. The *PhTPS1* RNAi construct was generated by using the Sol Genomics Network VIGS Tool (http://vigs.solgenomics.net/)[36] to identify the best target region of the *PhTPS1* coding sequence and to verify that the designed *PhTPS1* double-stranded RNA (dsRNA) trigger would not result in off-target interference. The *PhTPS1* RNAi construct included two spliced *PhTPS1* complementary DNA fragments corresponding to nucleotides 937–1,436 and 937–1,237 (in the antisense orientation) to create a hairpin structure. The sequence was then synthesized by Genscript (Piscataway) with flanking *AttL1* and *AttL2* sequences for LR recombination (Thermo Fisher Scientific) into the pB2WG7 binary vector under the control of the CaMV 35S promoter. *PhTPS1* RNAi transgenic plants were generated via *Agrobacterium tumefaciens* (strain GV3101)-mediated transformation using the standard leaf disk transformation method[37] and 16 transgenic lines were screened for *PhTPS1* mRNA in the flower tubes, sesquiterpene emission from the flower tubes, and accumulation of sesquiterpenes in pistils.

RNA isolation and qRT-PCR analysis. Total RNA was isolated from wild-type and *PhTPS1* RNAi flower organs collected at 14:00 at different developmental stages using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). Total RNA samples were treated with DNaseI (Thermo Fisher Scientific) and were reverse transcribed using the 5X All-In-One RT MasterMix (Applied Biological Materials). Gene expression was analyzed by qRT-PCR using gene-specific primers (Supplementary Table 1) and the $E^{\Delta Ct}$ method[38]. Expression levels were normalized to the petunia ortholog of the known *Arabidopsis* reference gene *Protein Phosphatase 2A-like*[39,40].

Metabolite profiling of internal pool and emission of terpenes. For the analysis of the internal pool of terpenes at different developmental stages, flower organs from 3 to 5 flowers per biological replicate were collected at 14:00 and crushed in 3 ml hexane. Naphthalene (2 nmol) was added as an IS. Samples were vortexed for 20 s, sonicated for 10 min and centrifuged at 2,000*g* for 4 min. Supernatant was recovered, concentrated under nitrogen to approximately 200 µl and analyzed by gas chromatography–mass spectrometry (GC-MS) (see below).

For analysis of glycosides, tissues were collected at 14:00, crushed in MeOH and extracted overnight at −20 °C. After sonication for 10 min, samples were centrifuged at 2,500*g* for 5 min. Supernatant was recovered and split into two equal parts, before drying in a Speedvac (Labconco). Both samples were resuspended in 500 µl phosphate-citrate buffer (100 mM, pH 5) and 100 µl Viscozyme L (Sigma-Aldrich) was added to only one sample. Both samples were overlaid with 500 µl of hexane containing internal standard and incubated at 37 °C overnight with gentle shaking (120 rpm). The organic phase was then recovered and extraction was repeated with an additional 500 µl hexane. Hexane extracts from each sample were pooled together, concentrated under nitrogen to approximately 200 µl, and analyzed by GC-MS (see below).

For the analysis of the effect of petunia tube on the terpene internal pools in pistils, tubes were removed from young flowers with scissors 4 d before anthesis. Naked pistils were covered with Eppendorf tubes to prevent desiccation. Sesquiterpene internal pools were analyzed in pistils after 4 d, which corresponds to day 1 of petunia flower development, as described above.

For the analysis of terpene content on pistil surface versus internal pools, pistils collected on day 1 post anthesis were first dipped for 30 s in 1 ml hexane to extract cuticular terpenes, and then subjected to extraction of the remaining internal terpene pools, as described above. Both samples were analyzed by GC-MS in parallel. Concurrent extraction of total terpenes from intact pistils was used as a control to ensure complete extraction during separate analysis of terpenoids on surface versus inside pistils. Each biological sample consisted of five pistils.

Petunia tube terpene emission was analyzed by dynamic headspace collection of volatiles starting at 12:00 on day 0 before anthesis. Tubes with reproductive organs and petal limbs removed were placed in 5% sucrose solution in a sealed 1 l glass jar with an inlet and an outlet. A cartridge containing 200 mg of Poropaq Q (80–100 mesh) (Sigma-Aldrich) was placed in the inlet to purify incoming air. A volatile collection trap (VCT) filled with 50 mg of Poropak Q was inserted in the outlet. Volatiles were trapped on the VCT at a flow rate of 100 ml min^{-1} for 24 h. VCTs were eluted with 200 µl of dichloromethane containing 2 nmol naphthalene as an internal standard before GC-MS analysis.

For the analysis of terpene emission from the inner (adaxial) and outer (abaxial) surfaces of the petunia tube, tubes on day 0 before anthesis were harvested at 12:00 h, removed from their reproductive organs, fully opened to render the surface flat and placed in glass vials in a 5% sucrose solution. For Stir Bar Sorptive Extraction (SBSE), magnetic twister coated with Polydimethylsiloxane (PDMS) (Gerstel, Germany) were placed on the top of both sides of the tube and vials were closed. After 24 h, Twisters were eluted with 150 µL dichloromethane containing 2 nmol of internal standard and samples were analyzed by GC-MS. Emission of each terpene from the inner and outer side of the tube was presented as percentage of total emission of each terpene from both sides.

Heterologous expression of TPSs in yeast. To obtain full-length coding sequence (CDS) of petunia TPSs, total RNA was extracted from tubes (for *PhTPS1*) or pistils (for *PhTPS2*, *PhTPS3*, and *PhTPS4*) of flowers collected at 14:00 on day 0 before anthesis using the Spectrum Plant Total RNA Kit. After treatment with DNase I (Thermo Fisher Scientific), cDNAs were synthesized from 2 µg of total RNA with SuperScript III reverse transcriptase and oligo(dT)18 primers (Thermo Fisher Scientific) according to the manufacturer's protocol. Vector constructs were generated as described previously[5] by using the USER cloning method (New England Biolabs) as described in ref. [41]. USER extensions were added to the gene-specific primers (Supplementary Table 1) for subcloning the *PhTPS1*, *PhTPS2*, *PhTPS3*, and *PhTPS4* coding sequences into the yeast expression plasmid pYeDP60u2.

To identify product specificity of sesquiterpene synthase candidates, the yeast expression constructs containing *PhTPS1*, *PhTPS3*, and *PhTPS4* coding sequences were transformed into the WAT11 yeast strain. Yeast were cultured according to the methods described in ref. [42], with a few modifications as follows. After verification by PCR, individual transformed colonies were grown in selection medium overnight, then diluted tenfold in 250 ml complete media and grown for another 30 h before induction with galactose (20 g $^{-1}$). Induced cultures were poured into a 1 l glass bottle. An inlet tube was submerged in the culture to provide aeration, and a vacuum line with a VCT containing 200 mg of Poropak Q (80–100 mesh) was inserted in the outlet to trap produced terpenoids. The culture was stirred with a magnetic bar, and VOCs were collected for 2 d. Terpenoid products were eluted from the VCTs with 1 ml dichloromethane every 24 h and analyzed by GC-MS. To determine product specificity of the monoterpene synthase candidate, the yeast expression construct containing *PhTPS2* coding sequence was transformed into the K197G yeast strain. Yeast were cultured according to the methods described in ref. [43], with the same modifications as mentioned above, and VOC products were analyzed as above for the sesquiterpenes.

GC-MS analysis. GC-MS analysis was performed on an Agilent 7890B gas chromatograph (Agilent Technologies) equipped with a HP-5MS column (30 m, 0.25 mm, 0.25 µm; Agilent Technologies) and coupled to an Agilent 5977B high efficiency electro impact mass spectrometer (Agilent Technologies). Sample (2 µl) was injected at 1:10 split using a Gerstel cooled injector system (CIS4) with an injection gradient of 12° s^{-1} from 60 to 250 °C. Column temperature was held at 50 °C for 0.5 min, then heated to 320 °C (held for 5 min) at 20 °C min^{-1}. Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. MS ionization energy was set at 70 eV, and the mass spectrum was scanned from 50 to 300 amu. For terpene profiling, chromatograms were analyzed with AMDIS software (http://www.amdis.net/) for mass spectra deconvolution using extracted ion chromatograms of specific ions 69 *m/z* (geraniol), 93 and 161 *m/z* for mono- and sesquiterpenes and 128 *m/z* for internal standard, naphthalene. Products were identified by comparison of mass spectra to the NIST/EPA/NIH Mass Spectral Library (version 2.2). Quantification of terpenes was performed using the Mass Hunter quantitative software (Agilent Technologies) using response factors relative to the internal standard determined experimentally for the commercially available authentic standards germacrene D (representative sesquiterpene), geraniol (representative monoterpenol), and nerolidol (representative sesquiterpene alcohol) and normalized to the weight of the tissue.

Terpene synthase activities in crude extracts from petunia tubes and pistils. Of the top 1 cm of tubes or whole pistils without ovaries, 100 mg were colleted at 14:00 on day 0 before anthesis, frozen in liquid nitrogen, ground to a fine powder and resuspended in 1 ml of extraction buffer consisting of 50 mM 3-[N-morpholino]-2-hydroxypropanesulfonic acid, pH 6.9, 5 mM DTT, 5 mM $Na_2S_2O_5$, 1% [w/v] polyvinylpyrrolidone-40, and 10% glycerol, as described previously[44]. Extracts were shaken gently for 30 min on ice followed by centrifugation (15,000*g* for 20 min at 4 °C). The supernatant was concentrated using Amicon Ultra 0.5 ml Centrifugal Filters, Ultracel – 10 K (MilliporeSigma), desalted with Econo-Pac10DG Desalting Columns (Biorad) and resuspended in the assay buffer containing 50 mM HEPES, pH 7.2, 100 mM KCl, 7.5 mM $MgCl_2$, 20 µM $MnCl_2$, 5% (v/v) glycerol, 5 mM DTT. Protein concentration was determined by the Bradford method[45] with BSA as a standard. Activity assays were performed in a final volume of 500 µl assay buffer with 300 µg total protein and 100 µM (*E*,*E*)-FPP (*E*–*E*-farnesyl diphosphate) (Echelon Biosciences) as substrate. Assay mixture was overlayed with 500 µl hexane and incubated for 1 h at 28 °C. Samples were extracted by adding 1 ml of hexane containing 2 nmol of internal standard and vortexing. The organic phase was recovered, concentrated under nitrogen gas to about 200 µl and analyzed by GC-MS.

Labeling experiments. To track the tube-synthesized sesquiterpenes in pistils, wild-type flowers were collected at day 0 before anthesis at 14:00, the tube was cut along the length and after removal of reproductive organs was placed in 1 ml 1% sucrose containing 5 mg of [2-^{13}C]-mevalonolactone in 20 ml scintillation vials (see the experimental setup in Supplementary Fig. 10b). For each pistil, one-third of the style was removed and the pistil was placed in a microvial with 5% sucrose before returning back to the flower tube. The microvial ensured that pistils were not in contact with the labeled precursor. After 24 h, sesquiterpenes were extracted from

both tubes and pistils, and analyzed by GC-MS as described above. The amount of unlabeled and labelled sesquiterpenes was determined based on the specific ions 161 *m*/*z* (M + 0) and 162 + 163 *m*/*z* (M + 1 and M + 2), respectively.

Exogenous fumigation. To analyze the capacity of pistils to absorb exogenous sesquiterpenes, pistils were collected on day 0 before anthesis at 14:00 and placed in a small container with 1 ml 5% sucrose solution inside 20 ml scintillation vials. A filter paper spotted with 10 µl of 1 M caryophyllene (10 µmol) in methanol was placed in the scintillation vials, which were then capped. Three pistils were used for each biological replicate. Pistils were collected at the indicated time points after treatment and internal pools were extracted and analyzed as described above. To determine the relative ability of pistils, stamens and tubes to absorb sesquiterpenes, flowers were collected at day 0 before anthesis at 14:00, the tubes were opened with a blade along their length so that that each organ was exposed to the air, and placed in a small container with 1 ml 5% sucrose solution inside a 20 ml scintillation vials containing a filter paper spotted with 10 µl of 1 M caryophyllene (10 µmol) as above. After 5 h of treatment, each organ was collected, extracted, and analyzed for internal pools of caryophyllene as described above.

Analysis of pistil parameters and seed production. Pistils from wild-type and *PhTPS1* RNAi lines were collected on day 1 post anthesis at 14:00 and weighed. The stigmas were dissected for imaging on a Leica MZ FLIII fluorescence stereo microscope (Leica Microsystems). Major and minor axis lengths (see details in Supplementary Fig. 14d) were determined using ImageJ version 2.0.0-rc-68/1.52e. To measure the style diameter, hand sections were made just under the stigma, then imaged and analyzed as described above. To measure seed production, flowers from wild-type and *PhTPS1* RNAi lines were emasculated on day 1 post anthesis and cross-pollinated at 18:00 on day 2 post anthesis. At 4 to 5 weeks after pollination, mature seed pods (brownish in color) were placed in a 12 ml plastic tube. After pods cracked, seeds were collected and dried at 37 °C for 2 d. The weight of total seeds per pod was determined, and 120 seeds per pod were counted out and weighed to determined seed weight and total number of seeds per pod.

Microbiome profiling. Pistils were removed from flowers using sterile forceps, taking care to ensure that pistils did not touch any other plant tissue. Pistils of wil- type and *PhTPS1* RNAi-11 plants were collected on day 0 before anthesis and day 2 post anthesis at 14:00 and placed in ZR BashingBeads Lysis tubes containing 750 µL of ZymoBIOMICS lysis solution (ZymoBIOMICS DNA Miniprep Kit, Zymo Research Corporation). Lysis tubes were shaken (20 Hz, 5 min) using a TissueLyser II bead mill (Qiagen) to extract DNA from microbes while leaving plant tissues undamaged. Microbial DNA was purified using the same kit, following the manufacturer's instructions. Microbiome profiling of isolated DNA samples was performed by Eurofins Genomics. Eurofins Genomics amplified and Illumina MiSeq sequenced the V3V4 region of the 16S rRNA gene to identify bacterial OTUs (and the ITS2 gene for fungal strains) following the manufacturer's instructions (InView—Microbiome Profiling 3.0 with MiSeq). Sequences were demultiplexed, the primers were clipped, forward and reverse reads were merged, and merged reads were quality filtered. Microbiome analysis was performed by Eurofins Genomics using the company's standard procedure (the following description of analysis is provided by Eurofins Genomics): reads with ambiguous bases ('N') were removed. Chimeric reads were identified and removed based on the de-novo algorithm of UCHIME[46] as implemented in the VSEARCH package[47]. The remaining set of high-quality reads was processed using minimum entropy decomposition (MED)[48,49]. MED provides a computationally efficient means to partition marker gene data sets into OTUs. Each OTU represents a distinct cluster with significant sequence divergence from any other cluster. By employing Shannon entropy, MED uses only the information-rich nucleotide positions across reads and iteratively partitions large data sets while omitting stochastic variation. The MED procedure outperforms classical, identity-based clustering algorithms. Sequences can be partitioned based on relevant single nucleotide differences without being susceptible to random sequencing errors. This allows a decomposition of sequence data sets with a single nucleotide resolution. Furthermore, the MED procedure identifies and filters random 'noise' in the data set; that is, sequences with a very low abundance (less than 0.02% of the average sample size). To assign taxonomic information to each OTU, DC-MEGABLAST alignments of cluster representative sequences to the sequence database were performed (reference database: NCBI_nt (Release 2018-07-07)). A most specific taxonomic assignment for each OTU was then transferred from the set of best-matching reference sequences (lowest common taxonomic unit of all best hits). A sequence identity of 70% across at least 80% of the representative sequence was a minimal requirement for considering reference sequences. Further processing of OTUs and taxonomic assignments was performed using the QIIME software package (version 1.9.1, http://qiime.org/)[50]. Abundances of bacterial taxonomic units were normalized using lineage-specific copy numbers of the relevant marker genes to improve estimates[51].

Given the low diversity of bacterial OTUs in the samples and the high number of sequences that were assigned as originating from plant tissues, we compared the microbiome of the pistils to the floral microbiome of a *Brassica rapa* plant that was cultivated from surface sterilized seeds under sterile conditions and of a *Brassica rapa* plant cultivated in soil in the laboratory (Supplementary Dataset 1). For detailed methods see ref. [23]. The microbiome of the flowers of plants cultivated from surface-sterilized seeds consisted of 3 OTUs, whereas the microbiome of the 2 samples of non-sterile flowers was more diverse with 68 and 115 OTUs. As most samples contained an OTU belonging to the genus *Delftia*, which is probably a contaminant, these OTUs were removed from analysis. The other OTUs found to be associated with petunia pistils were not found in sterile *Brassica* samples. The comparison of the microbial communities of the wild-type and *PhTPS1* RNAi-11 petunia flowers with the microbial communities of *B. rapa* indicates that the relatively low number of reads assigned to bacterial OTUs in the petunia samples reflects the low diversity and abundance of bacteria on these surfaces rather than a methodological artifact. However, further experiments may be required to fully characterize the microbiome of petunia pistils. Fastaq files of samples containing the sequences of the OTUs associated with *Petunia* and *Brassica* are deposited at the European Nucleotide Archive (PRJEB29416 (ERP111715)).

Analysis of pollen tube growth. Flowers from wild-type and *PhTPS1* RNAi-11 lines were emasculated on day 1 post anthesis and cross-pollinated at 18:00 on day 2 post anthesis. The pollinated pistils were then collected at 5 h, 15 h, 25 h, and 36 h after hand pollination and fixed immediately in ethanol-acetic acid (3:1 v/v) for 2 h. After clearing in 8 N NaOH at room temperature for 24 h, they were stained using aniline blue (0.1% aniline blue in K_3PO_4) for 24 h (ref. [52]). Four pistils per sample were analyzed for each time point and images were captured with a Nikon Eclipse Ti2-E microscope (Nikon Instruments).

Complementation assays. For gas phase complementation of terpenes in *PhTPS1* RNAi pistils, flowers from wild-type and *PhTPS1*-RNAi-11 lines were collected on day 0 before anthesis at 12:00, the tube was cut along the length and after removal of reproductive organs was placed in 1 ml 1% sucrose in 20 ml scintillation vials (see experimental setup in Supplementary Fig. 10c). For each pistil, one-third of the style was removed and the pistil was placed in a microvial with 5% sucrose before returning back to the flower tube. Wild-type pistils were placed in wild-type tubes, and *PhTPS1* RNAi-11 pistils were placed either in wild-type tubes or *PhTPS1* RNAi-11 tubes. After 24 h, sesquiterpenes were extracted from pistils and analyzed by GC-MS as described above.

To complement the development of *PhTPS1* RNAi-11 stigma, pistils from wild-type and *PhTPS1*-RNAi-11 lines were harvested 4 d before anthesis at 12:00, and grown in vitro in Magenta boxes containing Murashige and Skoog (MS) medium basal salt (Sigma-Aldrich) supplemented with 3% sucrose, 0.01 mg l^{-1} thiadiazuron and 0.1 mg l^{-1} gibberellic acid (see the experimental setup in Supplementary Fig. 10d). In parallel, wild-type and *PhTPS1* RNAi-11 line flowers were collected on day 0 before anthesis at 12:00, surface sterilized for 2 min with 50% commercial bleach and rinsed several times with sterile water. Tubes were cut along their length and after removal of reproductive organs were placed carefully around the pistils in MS media. Wild-type tubes were placed around wild-type or *PhTPS1* RNAi-11 pistils, and *PhTPS1*-RNAi-11 tubes were placed around wild-type or *PhTPS1* RNAi-11 pistils. Tubes were replaced every 48 h. In vitro reassembled flowers were cultivated at 20 °C under fluorescent light (35 µE m^{-2} s^{-1}) with a 16 h/8 h light/dark photoperiod. After 4 d, pistils were collected, weighed, and the stigma and style parameters (see details in Supplementary Fig. 14d) were measured using a microscope as described above.

Quantification of total wax from tube and pistil. Wild-type tubes, pistils, and stamen were collected on day 0 before anthesis at 14:00 and separately submerged in glass scintillation vials each containing 5 ml of hexane. Vials were vortexed for 30 s and the solvent was decanted into clean scintillation vials. Hexane extracts were subsequently dried to completion under a gentle stream of nitrogen gas. Dried wax residues were then weighed to quantify the total wax coverage on individual floral components. Each biological replicate consisted of floral organs collected from 12 flower buds on day 0.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Plant material generated in this study is available from the corresponding author upon request. For the microbiome, Fastaq files of samples containing the sequences of the OTUs associated with *Petunia* and *Brassica* are deposited at the European Nucleotide Archive (PRJEB29416 (ERP111715)). The sequences reported in this paper have been deposited in GenBank database with the following accession numbers MK159027 for PhTPS1, MK159028 for PhTPS2, MK159029 for PhTPS3, and MK159030 for PhTPS4.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	GC-MS data were collected and analyzed using Agilent MassHunter Workstation Software v.B. 07.01. qRT-PCR data were collected using StepOne v.2.2.2.
Data analysis	Non metric multi dimension scalings (NMDS) was performed using R v. 3.5.0. GC-MS data were collected and analyzed using Agilent MassHunter Workstation Software v.B. 07.01. Excel 365 and GraphPad Prism version 8.0.0 were used to analyze data, MEGA 7 version 7.0.18 was used for phylogenetic analysis, Cufflinks version 2.0.2 and edgeR package (v. 3.0.3) were used to measure gene expression in RNAseq data sets, AMDIS Version 2.73. Build 2017/04/25 was used to identify unknown compounds on GC chromatograms, the Sol Genomics Network VIGS Tool was used to design the PhTPS1-RNAi sequence: http://vigs.solgenomics.net/, QIIME software package version 1.9.1 was used for OTUs and taxonomic assignments, ImageJ version 2.0.0-rc-68/1.52e was used for microscopy analysis.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All figures except for Fig 4 and Supplementary Fig.10 have associated raw data available upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	A minimum sample size of 3 independent biological experiments was chosen to obtain valid statistical analyses.
Data exclusions	No data exclusion
Replication	At least three biological replicates were performed for each experiments and all attempts at replication were successful.
Randomization	Plants were randomized in greenhouse, and sample orders were randomized during analysis.
Blinding	Blinding is not necessary because results are qualitative for all experiments, and not subjective. Blinding was not possible in these experiments, as samples were analyzed immediately after collection, thus the treatments were known to the investigator.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging